

Research Article - Histology and Cell Biology

## Effect of vitrification on morphology and in-vitro maturation outcome of human immature oocytes

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### Summary

**Background.** In assisted reproductive techniques, 85% retrieved oocytes are mature, and the rest are immature. These immature oocytes may be matured in vitro, and used in subsequent in vitro fertilization program. The purpose of this study was to determine the maturation capacity and morphology of human immature oocytes in both fresh and vitrified-thawed, in vitro matured oocytes with regard to the maternal age and cause of infertility.

**Materials & Methods.** The first group of immature oocytes (n=103) were directly matured in vitro (fIVM), and the second group (n=102) were vitrified and stored in liquid nitrogen. After thawing, the samples underwent in vitro maturation (vIVM). Oocyte maturation was assessed by the presence of the 1<sup>st</sup> polar body and pronuclei. After 48 h incubation, each matured oocyte was assessed for ooplasm color, perivitelline space normality and shape regularity.

**Results.** After retrieval, 27% oocytes were immature (9.5 % metaphase I and 17.5% germinal vesicle stage). The rate of maturation of fIVM (61.2%) was significantly higher than that of vIVM (33.3%). The percentage of maturation in women under age of 30 was higher in both fIVM and vIVM. The maturation rate after IVM was higher in patients with male infertility than in those suffering of ovarian infertility.

**Conclusion.** Vitrification is a suitable technique for preservation of immature oocytes, especially at the germinal vesicle stage, in stimulated ovarian cycles. It should be noted that the maturation outcome of oocytes at germinal vesicle stage was better than that of metaphase I oocytes. Therefore, we recommend vitrifying germinal vesicle stage oocytes for subsequent in vitro maturation.

### Key words

Germinal vesicle stage; meiotic metaphase I; male infertility; female infertility; ovarian hyperstimulation.

### Key to abbreviations

fIVM: fresh in-vitro maturation

vIVM: vitrification plus in-vitro maturation

GV: germinal vesicle

M1: meiotic metaphase 1

PVS: perivitelline space

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## Introduction

In vitro maturation (IVM) of immature oocytes has been proposed as an alternative for conventional in vitro fertilization (IVF) treatment in certain cases. IVM has several advantages of reducing costs, avoiding the side effects of ovarian hyperstimulation syndrome and simplifying treatment for certain infertile couples (Al-Hasani *et al.*, 2007; Liu *et al.*, 2010; Ben-Ami *et al.*, 2011). Thus, IVM would avoid the high amounts of exogenous gonadotropins required for controlled ovarian hyperstimulation.

During the last decade, considerable progress has been made in the development of protocols for cryopreservation of mature oocytes. Also, there are practical applications for freezing immature oocytes, including fertility preservation for young cancer patients or patients without a male partner and contra-indication to ovarian stimulation in cases of hormone-sensitive tumors (Fuchinoue *et al.*, 2004; Cobo *et al.*, 2008; Homburg *et al.*, 2009). Immature oocyte cryopreservation may also prove beneficial during routine assisted reproductive techniques in combination with IVM. The use of these oocytes for cryopreservation may circumvent some of the limitations associated with freezing of mature oocytes, specifically relating to the functional integrity of the meiotic spindle and ploidy of resulting embryos (De Vos *et al.*, 1999; Yoon *et al.*, 2001; Hreinsson and Fridström, 2004; Ben-Ami *et al.*, 2011). IVM before freezing may render the oocyte even more vulnerable to damages associated with cryopreservation, given that IVM per se undoubtedly imposes additional stress on the oocyte. Therefore, it is more logical to freeze immature oocytes prior to IVM (Wu *et al.*, 2001; Cao *et al.*, 2009).

Past clinical studies with germinal vesicle stage (GV) oocytes as well as vitrification protocols have documented wide variability of results for survival, maturation, fertilization, and pre-implantation embryo development (Moor *et al.*, 1998; Kim *et al.*, 2000; Wu *et al.*, 2001; Liu *et al.*, 2010; Ben-Ami *et al.*, 2011). However, there are limited reports of vitrification of human GV oocytes. Most recently, Fasano *et al.* (2010) reported, for the first time, a side-by-side comparison of GV oocyte cryopreservation by either slow-freezing or vitrification (Fasano *et al.*, 2010). Further investigations are therefore needed on immature oocyte cryopreservation, together with maturation evaluation. Vitrification is an attractive alternative to slow freezing methods. It requires both ultra rapid cooling rates and very high cryoprotectant concentrations to prevent ice crystal formation and to increase viscosity at low temperatures. A series of successful live births has been recently reported after IVM of vitrified-thawed oocytes (Mandelbaum *et al.*, 1988; Baka *et al.*, 1995). In contrast, at least one child was born with the use of slow cryopreserved GV oocytes that subsequently underwent IVM protocol before intracytoplasmic sperm injection (Fasano *et al.*, 2010).

The main objective of this prospective study was to investigate the impact of cryopreservation by vitrification on the morphology and maturation success of immature human oocytes at the GV and meiotic metaphase I (MI) stages.

## Materials and Methods

### Patients

A total of 89 infertile women, mean average age  $32.3 \pm 6.3$  years (range 19–41 years), were included in this cross-sectional study. The patients were stimulated in

view of intracytoplasmic sperm injection with the standard long-term stimulation protocol using human menopausal gonadotropin and human chorionic gonadotropin injections. The immature oocytes were donated for research, with consent, by patients of Yazd Institute for Reproductive Science. Protocols were approved by the ethic committee of the authors' institution. The oocytes were allocated in two groups: fresh-IVM (fIVM; n=103) and vitrified-IVM (vIVM; n=102). Infertile patients were divided into three etiology groups: I. female infertility (n=25); II. male infertility (n=43); and III endometriosis + male infertility (n=21). Simultaneously, they were also divided into two age groups:  $\leq 30$  (n=50) and  $> 30$  (n=39) years old.

### Immature oocyte retrieval

Oocytes were retrieved 36 h after 10,000 IU human chorionic gonadotropin (IBSA Co, Switzerland). Transvaginal ultrasound-guided puncture was used with a single lumen aspiration needle (Wallace, Smiths Medical International, UK), at a pressure of 150 mmHg. The collected oocytes were assessed for nuclear maturity under the stereo microscope (Olympus Co, Tokyo, Japan): cells in the germinal vesicle (GV) or metaphase I (MI) stages were considered immature. Oocytes with visible GV were considered in GV stage and those lacking both a polar body and a GV were considered at MI stage. After partial denudation with 80 IU hyaluronidase (Sigma Co, St. Louis, Mo, USA) and mechanical pipetting, the oocytes were assessed again for signs of maturity. Only the oocytes that were determined to be immature were used in this study. The first group of immature oocytes (n=103) were directly subjected to IVM (fIVM), and the second group of immature oocytes (n=102) were vitrified and stored in liquid nitrogen. After thawing, these oocytes also underwent IVM (vIVM). Before fIVM or vitrification, the morphology of all immature oocytes was evaluated with the aid of inverted microscope (Olympus). Both maturity and the morphological characteristics of oocytes were assessed again after IVM. In this study, evaluation of oocytes morphology was based on assessing: 1) ooplasm color; 2) perivitelline space (PVS) normality; and 3) oocyte shape. The total number of oocytes from 89 patients was 968 including both mature and immature oocytes. 73% of retrieved oocytes were mature (in meiotic metaphase II), while the rest were immature oocytes (9.5% MI and 17.5% GV).

### IVM of immature oocytes

Denuded oocytes were washed in 3 drops of culture medium, then cultured in IVM medium consisting of: Ham's F10 (Biochrom Co, Germany) supplemented with 0.75 IU LH, 0.75 IU FSH (Menogon, Ferring Co, Germany) and 40% follicular fluid (FF), as described previously (Wu *et al.*, 2001). Briefly, FF was centrifuged at 300 g for 10 min to remove blood and granulosa cells. After inactivation at 56 °C for 30 min, the clear FF was filtered with a 0.22  $\mu$ m filter and stored at -20 °C before use. The oocytes were evaluated for maturity after 48 h at 37 °C in an incubator with 5% CO<sub>2</sub> and 95% air, with high humidity. Oocyte maturation was assessed by the presence of the first polar body under a dissecting microscope.

### Vitrification and thawing of Immature Oocytes

Vitrification and thawing were performed according to a previously published protocol (Al-Hasani *et al.*, 2007). The immature oocytes were incubated in equilibration solution comprising of 7.5% ethylene glycol (EG) (Merck Co, Germany) and 7.5% dimethyl sulphoxide (DMSO) (Merck Co, Germany) in Ham's F-10 media supplemented with 20% human serum albumin (HSA) (Plasbumin Co, USA) for 5-15 min (depending on the time needed for re-expansion of the cell) at room temperature. After initial shrinkage and recovery, the oocytes were aspirated and placed into the vitrification solution (15% EG, 15% DMSO, 0.5 M sucrose) in Ham's F-10 medium supplemented with 20% HSA for 50-60 s at room temperature. During vitrification, after observing cellular shrinkage, oocytes were aspirated and placed on the tip of the Cryotops (Kitazato BioPharma Co., Japan), which were immediately transferred to liquid nitrogen. While in liquid nitrogen, the Cryotops were covered with caps and dropped into the freeze-canes. All the samples were finally stored into a nitrogen tank.

For thawing, the Cryotops were placed in thawing solution (1 M sucrose; Sigma) for 50-60 s at room temperature. They were then moved into 0.5 M sucrose for 3 min, followed by 0.25 M sucrose for 3 min at room temperature. All solutions were supplemented with Ham's F10 containing 20% HSA. The thawed immature oocytes were then rinsed 4-5 times in washing solution (Ham's F-10 + 20% serum). At the end, the oocytes were transferred into IVM medium in 50 $\mu$ l droplets. After 48 h incubation, maturity and morphology of matured oocytes were assessed.

### Statistical Analysis

Statistical analysis was performed using the SPSS (SPSS 16.0, Chicago, USA) statistical package. The data were analyzed using the chi-square test. P-value of <0.05 was considered statistically significant.

### Results

A total of 63 (61.2%) oocytes matured by fIVM, while the rate of maturation by vIVM was 33.3% (n=34) ( $P<0.001$ ; Table 1). The number of GV oocytes that matured in vitro was significantly higher for fIVM than vIVM oocytes ( $P<0.001$ ). On the contrary, the maturation rates of retrieved MI oocytes were similar between both groups. Fig. 1 presents the morphological characteristics of GV and MI oocytes, as well as a fIVM oocyte. The findings also demonstrated that vitrification-thawing did not have any significant impact on the morphological aspect of in-vitro matured oocytes, as the ooplasm color, PVS, and oocyte shape were comparable between the two groups (Table 2).

Table 3 presents the correlation of maternal age with oocyte maturation rates. The maturation rates in younger women were higher for fIVM. Conversely, in older patients, the oocyte maturation rate was higher for vIVM than fIVM. A total of 76, 105, and 24 immature oocytes were achieved from patients in groups I, II, and III, respectively. The highest maturation rate was recorded for the group with male infertility (49.5%). The maturation rates decreased in endometriosis+male infertility and ovarian infertility to 45.5% and 44.7%, respectively.

**Table 1** – Rates of in vitro maturation of fresh (fIVM) and vitrified oocytes (vIVM).

Groups	fIVM	vIVM	P value
Immature oocytes	63(61.2)	34(33.3)	<0.001
GV	47(68.1)	23(34.8)	<0.001
MI	16(47.1)	11(30.6)	NS

NS= not significant.

Values inside parentheses represent percent (%).

**Table 2** – Oocyte morphology before and after in vitro maturation of fresh and vitrified cells.

Groups	Before IVM			After IVM		
	Abnormal cytoplasm color	widePVS	Round oocyte	Abnormal cytoplasm color	Wide PVS	Round oocyte
fIVM	0	1	1.9	1.9	3.2	4.8
vIVM	0	1.7	2	50	11.8	14.8
P value	NS	NS	NS	<0.001	<0.001	<0.001

PVS = perivitelline space.

NS= not significant.

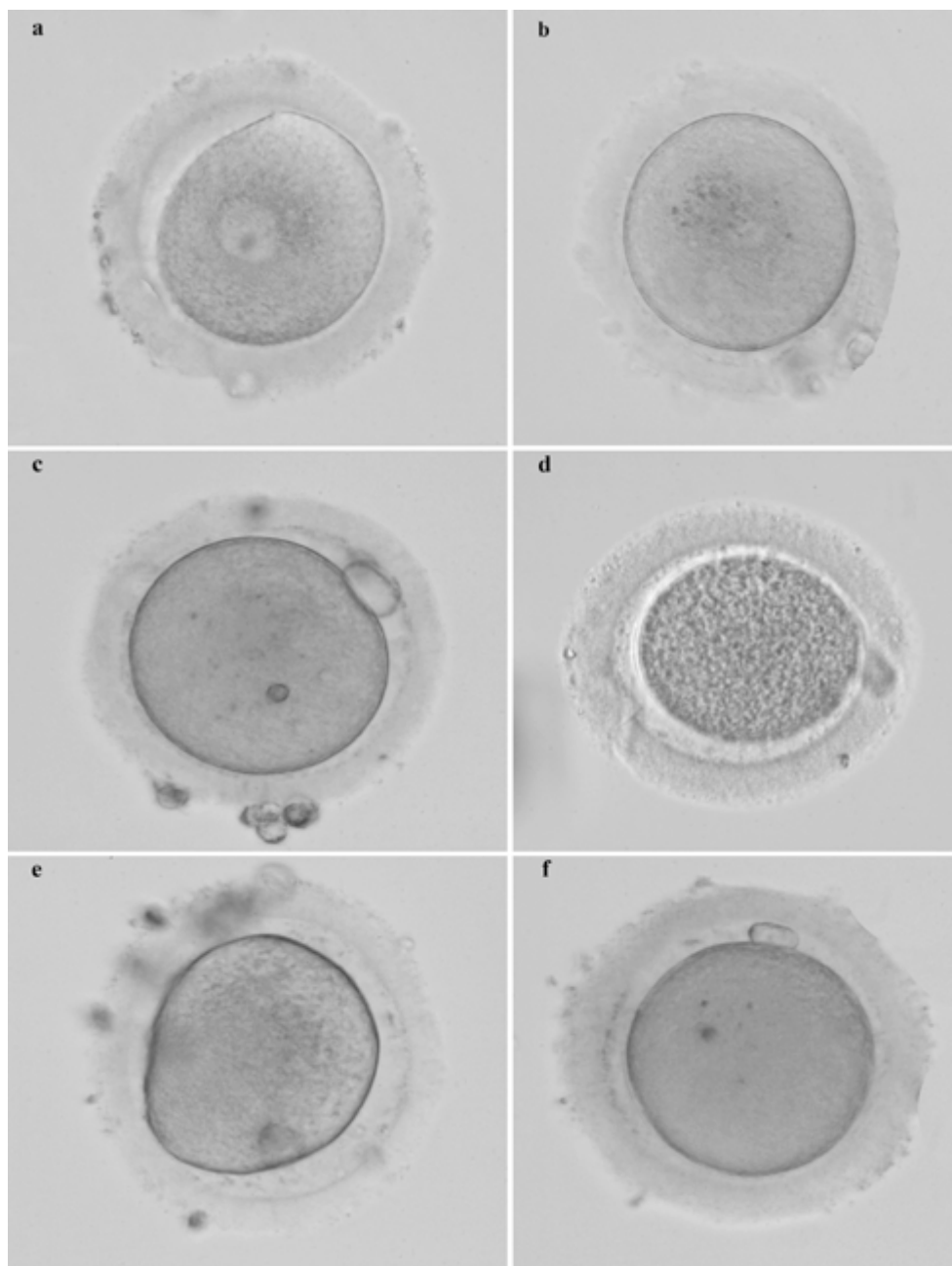
Values represent percent (%).

**Table 3** – Maturation rates of immature oocytes in different patient age groups.

Groups	fIVM		vIVM	
	Patient Age ≤30	Patient Age >30	Patient Age ≤30	Patient Age >30
Immature oocytes retrieved	61	42	37	65
Matured oocytes (%)	38 (62.3)	25 (59.5)	13 (35.1)	21 (32.3)

## Discussion

Oocyte freezing is a useful technique for assisted reproduction, particularly in cases of malignant diseases, premature ovarian failure, and oocyte donation (Ben-Ami *et al.*, 2011). Theoretically, GV oocytes are more resistant to cryo-injury because they have less depolymerization of the microtubules and are less prone to aneuploidy than mature oocytes (Cao *et al.*, 2009). Moreover, a greater number of immature than mature oocytes can be retrieved without ovarian stimulation. Therefore, cryopreservation of immature oocytes may become more useful than that of mature oocytes (Fuchinoue *et al.*, 2004; Al-Hasani *et al.*, 2007). During recent years, studies have shown that vitrification offers new interesting perspectives in the field of oocyte cryopreservation, appearing less traumatic than slow cooling (Cao *et al.*, 2009; Homburg *et al.*, 2009).



**Fig. 1** – Different oocyte aspects: a) Immature germinal vesicle (GV) oocyte, b) Immature metaphase I (MI) oocyte, c) In-vitro matured (MII) oocyte, d) dark oocyte, e) non-spherical oocyte, f) oocyte with wide perivitelline space (PVS).

In conventional IVF cycles, the remaining immature oocytes at the time of collection are not suitable for intracytoplasmic sperm injection. These immature oocytes, upon patient's agreement, are generally destined to research (Ben-Ami *et al.*, 2011). The present study has compared the maturation success of immature oocytes obtained from patients upon ovarian stimulation and subjected to fIVM and vIVM. It was demonstrated that 61.2% of the oocytes became mature after fIVM. This was similar to the data obtained by Nazari *et al.* (2011) who reported a 59.4% maturation rate for fIVM. Also, we found that when immature oocytes were vitrified and then matured in-vitro, a significant difference in maturation rate was seen when compared with fIVM. It has been shown that vitrification, although a successful cryopreservation technology, may have a negative effect on the outcome of oocyte maturation. Importantly, both groups of fIVM and vIVM showed that the maturation rates of GV oocytes were higher than those of MI oocytes. This could be related to chromosomal conservation within the membrane of GV. Also, microtubular structures are not yet formed in GV oocytes (Nazari *et al.*, 2011). In addition, we found that the rates of abnormal oocytes with ovoid shape, dark ooplasm, large and abnormal PVS were increased in vIVM oocytes. This indicates that cryopreservation may cause some injury to the morphological characteristics of oocytes. Therefore, more investigations are necessary in order to improve cryotechnology.

In this work, half of the oocytes survived vitrification-thawing, which is similar to the findings of Baka *et al.* (1995). However, other authors showed a lower survival rate (Mandelbaum *et al.*, 1988). Recently, Nazari *et al.* (2011) showed that almost 87% of cryopreserved oocytes could survive, but with increasing rates of vacuolization and darkening of ooplasm.

In addition, another important factor possibly influencing the maturation of human oocytes and investigated in this study is age. The data showed that there was no significant relation between patient's age and the rates of maturation of both vIVM and fIVM. Therefore, the maternal age did not influence the maturation rates of oocytes in our study. In a large cohort study, Wiser *et al.* (2011) reported that IVM is not suitable for infertile patients over the age of 40. On the basis of the present findings, on the contrary, IVM might be practicable for the majority of women seeking assisted reproduction.

In conclusion, our study showed that vitrification is a feasible technique for the cryopreservation of immature oocytes, particularly at GV stage, for subsequent application in clinical IVM. It is recommended, however, to use the MI oocytes in fresh IVM in order to increase the reproductive success, while vitrifying GV oocytes for subsequent IVM applications. Further studies are needed to limit the cell injury post vitrification.

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